MYCOBIOLOGY
2018, VOL. 46, NO. 3, 242–253
https://doi.org/10.1080/12298093.2018.1505245

RESEARCH ARTICLE

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The Major Postharvest Disease of Onion and Its Control with Thymol Fumigation During Low-Temperature Storage

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ABSTRACT

Onion (*Allium cepa* L.) is one of the major vegetable crops in Korea that are damaged and lost by pathogenic fungal infection during storage due to a lack of proper storage conditions. The aim of this study was to determine an appropriate control measure using thymol to increase the shelf life of onions. To control fungal infections that occur during low-temperature storage, it is necessary to identify the predominant fungal pathogens that appear in low-temperature storage houses. *Botrytis aclada* was found to be the most predominant fungal pathogen during low-temperature storage. The antifungal activity of the plant essential oil thymol was tested and compared to that of the existing sulfur treatments. *B. aclada* growth was significantly inhibited up to 16 weeks with spray treatments using a thymol solution. To identify an appropriate method for treating onions in a low-temperature storage house, thymol was delivered by two fumigation treatment methods, either by heating it in the granule form or as a solution at low-temperature storage conditions (*in vivo*). We confirmed that the disease severity was reduced up to 96% by fumigating thymol solution compared to the untreated control. The efficacy of the fumigation of thymol solution was validated by testing onions in a low-temperature storage house in Muan, Jeollanam-do. Based on these results, the present study suggests that fumigation of the thymol solution as a natural preservative and fungicide can be used as an eco-friendly substitute for existing methods to control postharvest disease in long-term storage crops on a commercial scale.

ARTICLE HISTORY
Received 12 February 2018
Revised 5 April 2018
Accepted 14 May 2018

KEYWORDS
Allium cepa; Botrytis aclada; fumigation; long-term storage; postharvest disease; sulfur; thymol

1. Introduction

Onions (*Allium cepa* L.) are an important seasonal crop in the world, and they are one of the commercially valuable crops used as a salad or seasoning vegetable in Korea. Therefore, the long-term storage of onions is necessary to ensure a steady supply throughout the year. The main cause for the reduction in onion yields is the various fungal infections that appear during long-term storage. It is vital that onion diseases are diagnosed early and effective management strategies are implemented to reduce the loss of onions. Onion quality is associated with many factors such as disease and humidity, etc during cultivation, production, storage, and distribution. Despite the advances in production technology, the postharvest loss during storage is still a major problem. Onions suffer from many postharvest diseases such as black mold, blue mold, neck rot, brown rot, soft rot, and smudge, among which, black mold, blue mold, and gray mold are the predominant diseases that restrict the domestic and international trade of onions [1,2]. Among the *Botrytis* species that cause neck rot in onions, *B. aclada* (Fresenius), *B. allii* (Munn), and *B. byssoidea* (Walker) are considered exclusively associated with the symptoms of neck rot in onions [3].

At present, research on onion storage has focused on low-temperature storage and radiation and fumigation treatments. The most important factor for low-temperature storage houses is maintaining the temperature (0 °C), relative humidity (70–80%), and cleanliness of the storage environment.

During the low-temperature (below 4 °C) storage of onions, the frequency of infections from storage pathogens can be reduced to 10 to 20%. However, low-temperature storage requires a higher cost because the harvest time of onions is in the summer. Moreover, gray mold neck rot and soft rot can also grow between 0 and 5 °C. Therefore, new technologies that can economically and effectively extend the shelf life of onions are needed.

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Supplemental data for this article can be accessed here.

Generally, many farmers are using the curing treatment after harvest before storage, and then pesticides or sulfur treatment during storage. However, the current methods are labor-intensive, high cost, and require time. Moreover, the postharvest application of fungicides is limited because of adverse effects from pesticide residues on food as well as from resistant pathogens [4,5].

Essential oils (EOs) from plants are substances that are produced as a plant defense mechanism against pathogens [6]. Thus, essential oils are used to control the growth of postharvest pathogens found on fruits and vegetables.

Thymol is the main component of thyme essential oil (p-cymene, 8.41%; thymol, 47.59%; γ-terpinene, 30.90%) obtained from the leaves of *Thymus vulgaris*, and pure thymol has shown approximately three-times stronger inhibition of microbial growth than that by the essential oil of thyme [7]. Thymol has been used in a wide range of products including cosmetics, oral cleaners, and preservatives within the limited range established by each product.

Thymol has been demonstrated as an antimicrobial agent in many previous studies [8–10]. However, most of the antifungal tests for postharvest pathogen control using thymol were performed at a laboratory scale, and scale-up experiments using a variety of thymol treatment methods are required for their effective application on farms and rural locations.

The objective of this work was to derive an effective treatment method using thymol, which shows antifungal effects against postharvest fungal diseases during low-temperature storage. In this study, the antifungal effect of thymol fumigation against onion postharvest fungal diseases was investigated at a commercial scale.

2. Materials and methods

2.1. Collection of onion bulb samples

Onion bulbs showing symptoms of rotting and discoloration were randomly selected from low-temperature storage houses in the Muan, Jeollanam-do province. Thus, 10–20 onion bulbs per storage house showing postharvest fungal infections were collected from five different storage houses (the size of the cold room was approximately 547 cubic meters) from Nonghyup ("National Agricultural Cooperative Federation"): Illo-Nonhyup, Mongtan-Nonhyup, Hyungkyung-Nonghyup, Maebong-Nonghyup, and Youngheung-Nonghyup.

2.2. Fungal isolates

For the isolation of fungal pathogens from the diseased onions stored in the low-temperature storage houses in the Muan region, three pieces from a portion with small lesion from each onion bulb were surface-sterilized with 70% ethanol for 3 min, followed by 1% (w/v) NaOCl for 1 min. The lesion pieces were washed three times with sterile distilled water (15 min each). The sterilized lesion pieces were lightly dried on a sterile filter paper and then transferred onto potato dextrose agar (PDA; Difco, USA) media at 25 °C for 4 days or at 4 °C for 4–12 days. Twelve onions from the Garak market in Seoul (the biggest market in Korea) were purchased and incubated in humid conditions for the growth of fungi. Twelve onion bulbs were incubated in 8.3 L plastic containers (three bulbs per container) laid with two layers of wet paper towels at 25 °C for 4 days or at 4 °C for 4–12 days. The part of onion grown with postharvest fungi (diameter of 5 mm) were transferred onto PDA and incubated at 25 °C. The isolated fungi were further purified with a mycelial tip culture using a stereomicroscope.

2.3. Molecular identification of fungal isolates

Well-grown fungal mycelia from the mycelial tip cultures were harvested with a sterilized toothpick and suspended in 0.5 mL of distilled water in a 1.5 mL centrifuge tube. These suspensions were centrifuged at 10,000 rpm for 10 min. After removal of the supernatant, genomic DNA was extracted with the Solg^{TM} genomic DNA Prep Kit (Solgent, Daejeon, Korea) according to the manufacturer's instructions.

Amplification of the 18S rRNA gene was performed with the primers ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR products were verified on 1.2% agarose gels stained with ethidium bromide. A PCR master mix containing Taq DNA polymerase, dNTPs, Tris-HCl, $MgCl_2$ stabilizer, and tracking dye was used according to the manufacturer's instructions (TaKaRa Bio Inc., Otsu, Japan). The PCR reaction conditions with 100 ng of template DNA were as follows: 30 cycles of 1 min. at 94 °C, 1 min at 55 °C, and 1 min and 50 s at 72 °C. The amplified fragments were recovered from the agarose gel using a gel extraction kit (Solgent).

For the detection of the more frequently isolated fungi during the low-temperature storage of onions, gene-specific PCR (GSP) primers previously described for detection of *B. aclada* in onions were used in this study. The oligonucleotide BA_F (5′-GTGGGGGTAGGATGAGATGATG-3′) [11] was used as the forward primer, and BA_R (5′-TTGAATTGGGAGAGCGTTCCTTCG-3′) was used as the reverse primer [12] to amplify a 200 bp DNA product. The nucleotide sequence of the PCR product was confirmed by sequence analysis using the same GSP primers (Macrogen, Seoul, Korea). These gene sequences of the fungal isolates were matched with those from a NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/). The experiments were performed in triplicate, all of which produced similar results. A phylogenetic tree from fungal isolates sequences was generated by UPGMA algorithm using the CLC workbench (version 8.0) software. Different annotated nucleotide sequences were aligned and phylogenetic trees were constructed. The nucleotide sequences of several species according to each fungal isolate strain were aligned. To assess the robustness of the topology, 1000 bootstrap replicates were performed by maximum parsimony.

2.4. Pathogenicity of the fungal isolates from the low-temperature storage

The fungi (*B. aclada, F. proliferatum, Penicillium* sp., and *R. oryzae*) isolated from the low-temperature storage houses in Muan were grown on PDA at 25 °C for 4 days or at 4 °C for 4–12 days. To confirm the pathogenicity of the fungi, fungal mycelia (with a diameter of 5 mm) from the edges of the growing cultures on the PDA were inoculated onto the outer and inner surfaces of onions sterilized with 1% (w/v) NaOCl. The onion skins were slightly scratched using sandpaper, and the inner surfaces were inoculated as half-cut bulbs using a flame-sterilized knife [13]. The inoculated onions were placed in a plastic box (2 L) containing two layers of wet paper towels to maintain the moisture and then incubated at 25 °C for 4 days or at 4 °C for 4–12 days. Five onions per treatment were conducted for each of the three replicate experiments. The lesion diameter was measured according to the incubation time and incubation temperature for all tested isolates.

2.5. The effects of thymol and sulfur on inhibition of the fungal growth in vitro

Thymol concentrations were selected based on previous studies as the optimal concentration with antifungal activity [14]. Thymol ($C_{10}H_{14}O$, Daejung Chemicals & Metals Co., Siheung, Korea) and sulfur powder (Duksan Pure Chemicals Co., Anseong, Korea) at a concentration of 30 mg L^{-1} and 20 mg L^{-1}, respectively, were dissolved in 100% ethanol, and then pipetted onto a paper disc (diameter: 8 mm). The paper disc was placed onto the center of PDA and then sealed; the petri dish was then incubated upside-down. Thymol/sulfur solution evaporated slowly with the incubation lasting more than 7 days at 24 °C. The method used for the fumigation by thymol vapor has been fully described previously [14,15].

2.6. Effect of spray of thymol solution on *B. aclada* infections in onions

Thymol concentration was chosen based on previous studies as the optimal concentration with antifungal activity [14]. Thymol at a final concentration of 30 mg L^{-1} was dissolved in 100% ethanol and sprayed onto the surface of onions. For the control, the ethanol without thymol and only sterilized distilled water (the untreated control) were also sprayed onto the onion surfaces. There were 8–10 onions per plastic box (31.2 cm (L) × 22.0 cm (W) × 16.4 cm (H); Biokips, Seoul, Korea) depending on onion size, with three replicate experiments per treatment. The onions were surface sterilized with 1 ppm of chlorine, and the conidia suspensions (approximately 1×10^6/mL) of *B. aclada* were sprayed onto the surface of the onion. The boxes were stored for 9 weeks at 4 °C, and fungal infections were determined at 9 weeks after storage.

2.7. Effect of scale up and commercial-scale test of thymol on postharvest diseases of onion

In *in vitro* assays, ethanol at a small scale evaporates spontaneously with an appropriate period of time. However, ethanol requires more time for evaporation when scaled-up. If the ethanol evaporates too slowly in a large container, its effectiveness would be reduced due to the natural loss. Due to this phenomenon, thymol (as a granule form or dissolved in ethanol) was evaporated by heating using an electric heating apparatus (Duruco, Changwon, Korea) in a low-temperature storage container (4 °C), and performed at a concentration of 30 μg per mL for the scale-up test. Complete evaporation of thymol required several hours in the cold storage container (farmer owned, a size of approximately 14.2 tons; 185 cm (L) × 390 cm (L) × 197 cm (H)) located in Iksan, Jeollanam-do province for the low-temperature storage of onions. To compare thymol fumigation with sulfur, sulfur was also evaporated by heating sulfur powder using the electric heating apparatus in another low-temperature storage container at a concentration of 20 μg L^{-1}. Some Agri-cooperation companies in Korea burn sulfur for fumigation of cold storage rooms for onions (4 °C). Thus, 200 kg of onion stored in a cold storage container was fumigated by heating with 30 mg L^{-1} thymol granule or thymol after dissolving in ethanol using a fumigation apparatus (Duruco). The fungal disease incidence was determined at 4 months after storage.

Furthermore, we performed a commercial-scale test in the low-temperature storage house to confirm the disease suppression activity of thymol fumigation dissolved in ethanol. The direct fumigation of thymol granule by heating resulted in powder residue near the fumigation apparatus. Therefore, this option was excluded in the commercial-scale test. Thymol at a final concentration after fumigation (weight:volume of storage room after excluding the volume of onions on the pellet, 10 mg L^{-1}) was evaporated for 6 h using ten fumigation apparatus per cold room in a low-temperature storage house (Figure 1, a volume size of 547 ton storing roughly 160 MT of onions, which is 8,800 onion bags at 20–22 kg in size; 132 pallets of 3 m^3 in size; total coverage, approximately 402 m^3 space) located in Muan-eub, Jeollanam-do province. The fumigated thymol was exhausted slowly through the ventilation fans. The treated onion bags in the storage room were maintained at 0 °C for 9 months. There were 3 untreated rooms that were compared to the treated storage room. At three, six. and nine months after fumigation, about 400 onion bags (approximately 20–22 kg) in cold rooms were screened to determine the fungal infection rate based on the number of counted onions, showing the visual presence of fungal mycelium. We collected all the diseased samples, brought them into the laboratory, and plated them on PDA. The ones that were still growing were identified by microscopy and molecular methods if necessary.

2.8. Effect of thymol and sulfur fumigation on the postharvest root pathogen of onion in a scale-up test

To estimate the fungal sources as postharvest infection of onion, the effect of fumigation on the fungal population of the root was studied in a scale-up test as previously described. The onion root is often the major fungal infection starting point.

The roots of the sampled onions were cut using a sterilized knife and weighed at 4 months after storage. The onion roots from 10 onions weighed approximately 10–20 g per replicate experiment were kept in a 250 mL Erlenmeyer flask containing 100 mL sterile distilled water, suspended for 20 min, and diluted serially up to 10^{-7} dilution. Then, 1 mL of the fungal suspension dilution was mixed with PDA before it was solidified and plated with kanamycin, and then incubated at 25 °C for 3 days to determine the size of the fungal population. This experiment was repeated 3 times.

2.9. GC-MS analysis of the thymol residue

To determine whether the thymol gas was evenly diffused in the air or was adsorbed into a localized zone, we analyzed the residual concentration of the thymol attached onto the wall, floor, and air inside the container. The gas inside the container was collected in a tedlar bag (0.5 L; PK Lab, Seoul, Korea) using an air pump, and then 40 mL of collected gas were directly dissolved into 2 mL of acetyl acetated solution. Next, 2 μL of the thymol gas solution was used for the GC-MS analysis. The thymol absorbed onto the wall and floor was collected by attaching the plastic bag (20.5 cm (W) × 18.2 cm (L)), and it was directly dissolved in the acetyl acetated solution. This was the same amount that was used for the GC-MS analysis. GC-MS was performed with a Shimadzu GC-2010 gas chromatography instrument coupled with a Shimadzu QP2010 mass spectrometer. Compounds were separated on a fused silica capillary column Rtx-5MS (100% polymethylsiloxane, 30 cm × 0.25 mm i.d.,

Figure 1. Thymol fumigation in a commercial low-temperature storage room.

0.25 mm film thickness). The oven temperature program was initiated at 70 °C, held for 10 min, and then increased at a rate of 5 °C min^{-1} to 195 °C, and then increased at a rate of 10 °C min^{-1} to 300 °C and held there for 15 min. The spectrometers were operated in the electron-ionization mode; the scan range was 35–500 amu; the ionization energy was 70 eV, and the scan rate was 0.20 s per scan. The injector, interface, and ion source were kept at 250, 250, and 200 °C, respectively. Split injection (1 µL) was conducted with a split ratio of 1:10, and helium was used as the carrier gas at a flow-rate of 1.0 mL min^{-1}. All samples were normalized after automatic calibration by the thymol retention time of the standard curve.

3. Results

3.1. Identification of low-temperature fungal pathogens in onions

To identify the predominant fungal pathogens during low-temperature storage, we compared disease occurrences and types of fungal diseases in the Garak commercial market and low-temperature storage houses of Muan Agri-cooperation. A total of 55 fungal isolates were identified from the Garak commercial market: *Fusarium proliferatum* (36%), *Penicillium* sp. (20%), *Rhizopus oryzae* (11%), and *Aspergillus niger* (33%) (Table 1 and Figure 2). A total of 68 fungal pathogens were isolated and identified from diseased onion bulbs in the low-temperature storage houses in Muan: *B. aclada* (44%), *F. proliferatum* (29%), *Penicillium* sp. (21%), *R. oryze* (6%) (Table 1 and Figure 2). These fungal pathogens were isolated from a pool of infected onions at five different low-temperature storage house locations. When comparing the isolated fungi from both the Garak commercial market and the low-temperature storage houses, *A. niger* was not observed in the low-temperature storage houses. In contrast, *B. aclada* was only observed in the low-temperature storage houses. *B. aclada* was the predominant fungal pathogen most frequently identified in the low-temperature storage houses. PCR products (200 bp) were obtained using gene specific primer of *B. aclada* (Figure 3).

Table 1. Comparison of isolated fungi from the Garak commercial market and low-temperature storage houses in Muan.

	Frequency of occurrence (%)	
Isolated fungi	GC market[a]	LTS houses[b]
Botrytis aclada	ND[c]	44%
Fusarium proliferatum	36%	29%
Penicillium sp.	20%	21%
Rhizopus oryzae	11%	6%
Aspergillus niger	33%	ND

[a]Garak commercial market located in Seoul.
[b]Low-temperature storage houses in the Muan.
[c]ND: not detected.

3.2. Pathogenicity of low-temperature fungal pathogens of onions

The radial growth rate and pathogenicity of four fungi (*B. aclada, F. proliferatum, Penicillium* sp., and *R. oryzae*) isolated from the low temperature storage houses were confirmed depending on different incubation temperatures (25 °C and 4 °C) (Supplementary Table S1 and Figure S1). Especially, *R. oryzae* was the fastest growing mycelium at 25 °C. However, after incubation at 4 °C, *R. oryzae* did not grow while *B. aclada* showed vigorous growth compared to the other fungi (Figure 4). By comparing the pathogenicity test results depending on the incubation temperature, the typical low-temperature storage fungal pathogen was identified as *B. aclada* in this study.

3.3. Potent antifungal activity of thymol compared to sulfur in vitro

Fungal growth of *B. aclada* treated with 30 mg L^{-1} of thymol showed higher inhibition compared to the sulfur treatment (Figure 5). Thymol treatment showed significant inhibition for more than 3 weeks in the PDA (data not shown). In contrast, the sulfur treatment slowed down the growth rate of the fungi compared to the untreated control. However, the sulfur treatment was not as potent as the thymol treatments in inhibiting fungal growth. When treated with ethanol, the fungi grew a little more slowly compared to the untreated control; however, it did not significantly inhibit fungal growth compared to the thymol and sulfur treatments. Thus, our results demonstrate highly significant antifungal effects of thymol and sulfur treatments on fungal growth (Figure 5).

3.4. Antifungal effect of a thymol spray treatment against B. aclada on onions

We compared the disease suppression of thymol treatment *in vivo* in order to develop a spraying method for thymol as an alternative option to fumigation. The disease incidence of storage pathogens was significantly reduced following the thymol treatment compared to the untreated control and ethanol treatment (Table 2). The onion deterioration rate increased after 9 weeks in storage for the untreated controls. The disease incidence rate of the onions following each treatment is shown in Table 2. The disease incidence rate was 97%, 50%, and 27% in the untreated control, ethanol treatment, and thymol treatment, respectively (Table 2).

The disease incidence of *B. aclada* was low at low-temperature storage compared to room temperature storage. In addition, the disease incidence of the onions stored for 9 weeks at low-temperature was compared to the untreated controls (Supplementary Table S2). Thymol at 30 mg L^{-1} showed a significantly higher antifungal effect against the predominant fungal pathogen *B. aclada* on onions in the low-temperature storage houses.

3.5. Disease suppression of thymol versus sulfur fumigation

The demonstration test for the thymol and sulfur fumigation of *B. aclada* on onions in cold storage containers confirmed their significant antifungal

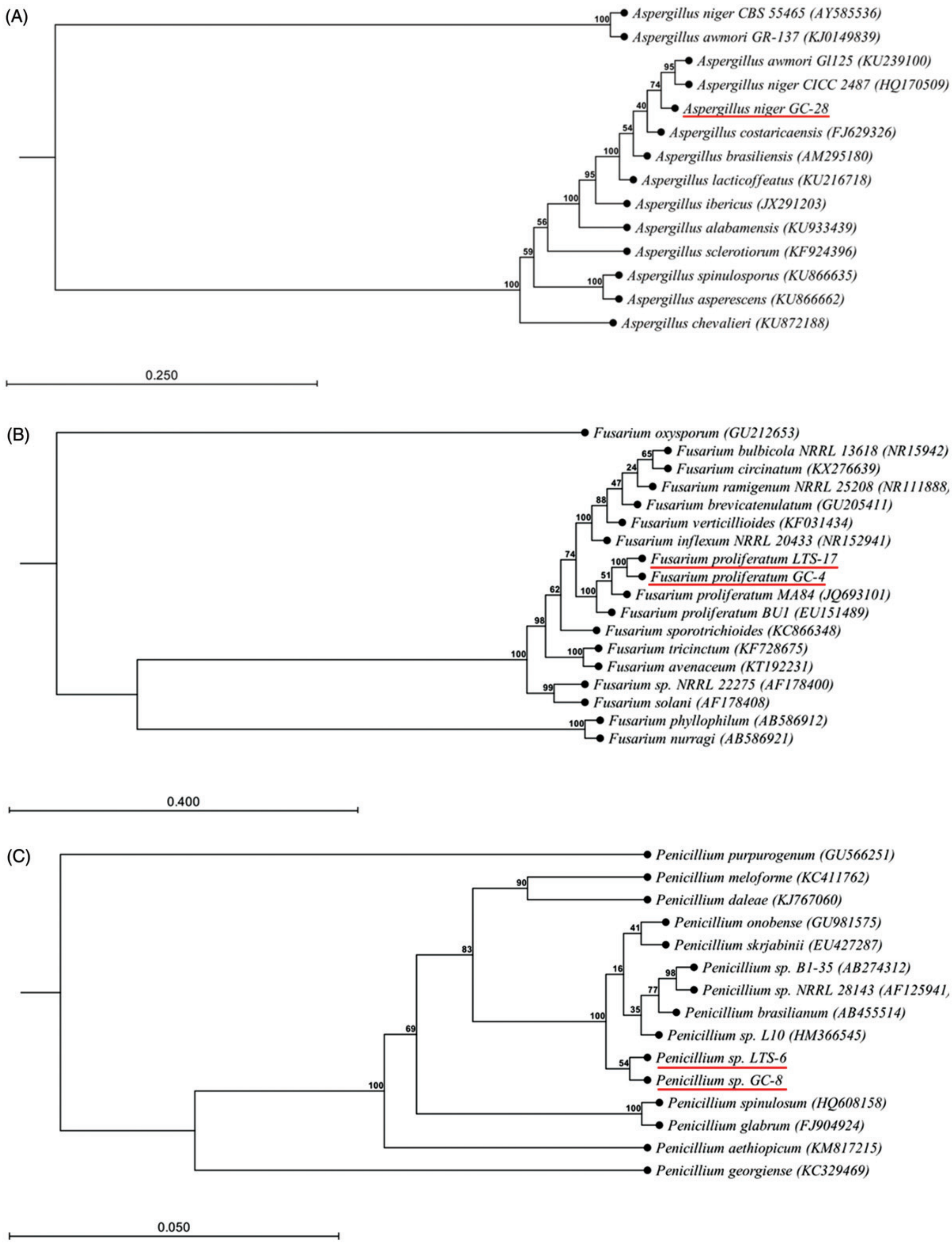

Figure 2. Phylogenetic trees constructed by the neighbor-joining method using DNA sequences of fungi isolated from the Garak commercial market and the low-temperature storage houses in the Muan area. (A) *Aspergillus niger*. (B) *Fusarium proliferatum*. (C) *Penicillium sp*. (D) *Rhizopus oryzae*. (E) *Botrytis aclada*.

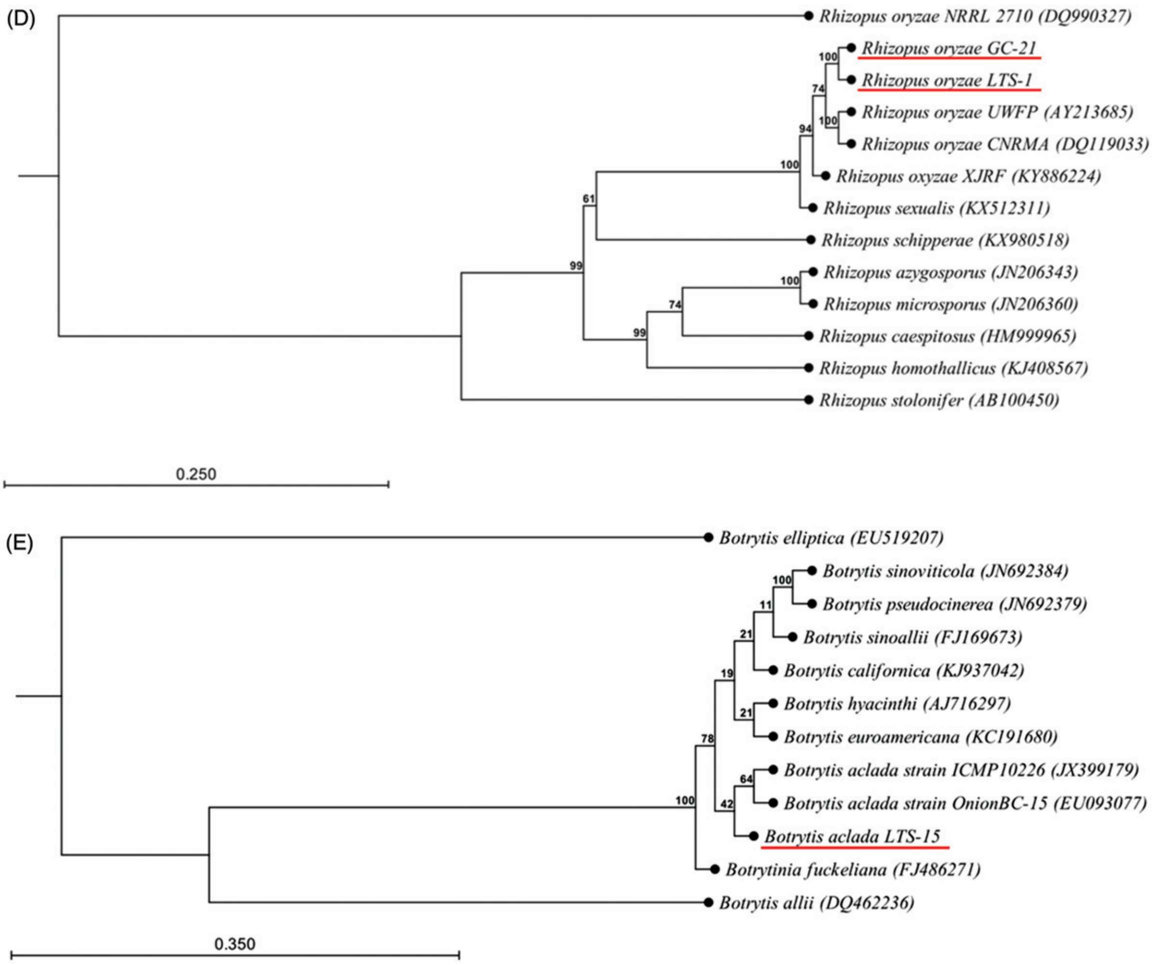

Figure 2. Continued.

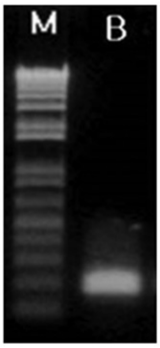

Figure 3. PCR using gene-specific primers for the detection of *B. aclada*. The PCR products (200 bp) of *B. aclada* were detected (M, 1 kb plus DNA marker; B, *B. aclada*).

effects. The disease incidence rate after the thymol treatment was significantly lower than that of the untreated control: 0%, 17.8%, and 33.3% for the thymol treatment, sulfur treatment, and untreated control, respectively (LSD, $p \leq 0.05$) (Table 3). Moreover, fungal growth was not observed in the onions treated with thymol for 16 weeks, although many onions showed fungal infection and sprouts in the untreated control onions (Supplementary Figure S2). Given these results, the thymol treatment was assumed to regulate the physiological activity while at the same time exhibit an antifungal activity.

3.6. Reduction of fungal population in onion roots by thymol fumigation

Most fungi penetrate through the roots or wounds of infected onions. In infected onion roots from cold containers, most of the onion roots were infected by the fungal pathogens as demonstrated by a fungal colony counting assay. In the onion roots infected by the fungal pathogens, the fungal population sizes of onion roots treated with sulfur and thymol (20 mg and 30 mg L^{-1}, respectively) were reduced by 3- and 12-fold, respectively, compared to the untreated control. In addition, the thymol treatment showed the best antifungal effect against the onion root fungi (Table 4).

3.7. The efficacy of thymol fumigation on postharvest disease of onions in commercial storage room and analysis of thymol residue

Fumigation (10 mg L^{-1} of thymol based on the total volume of the storage room excluding the onion volume on the pellet) using thymol to reduce the

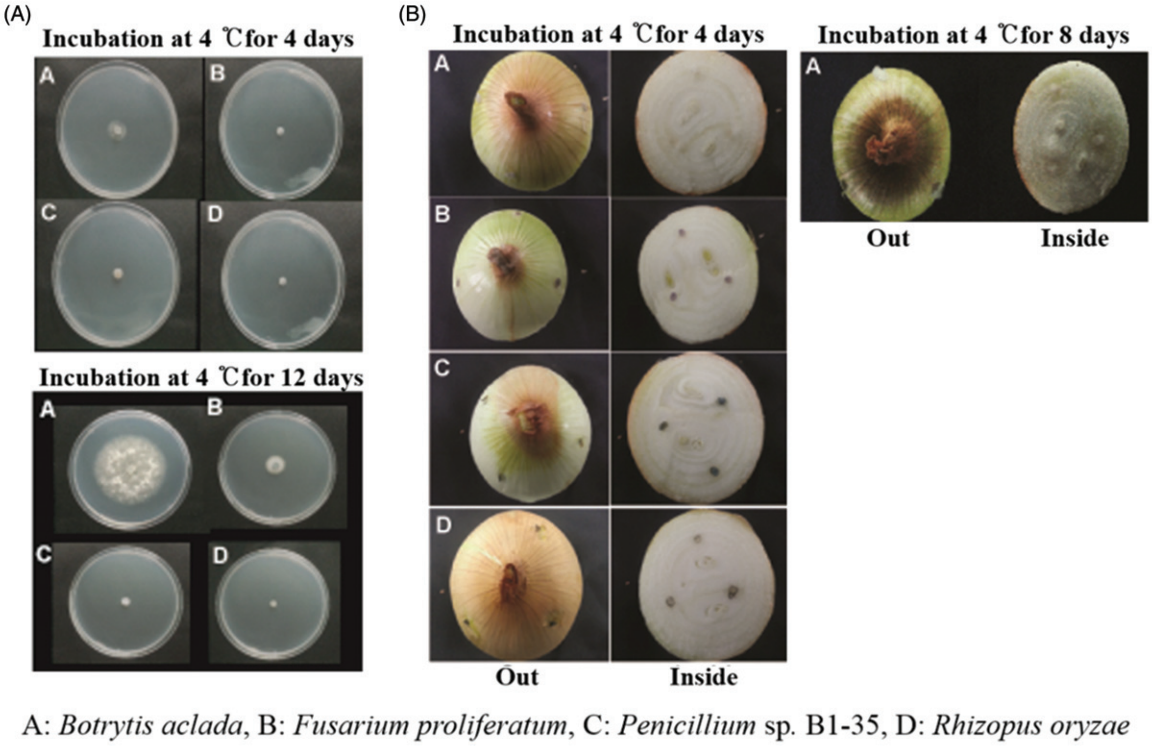

Figure 4. Identification of pathogens at low-temperature storage by the *in vitro* (A) and *in vivo* (B) pathogenicity tests. We confirmed that *B. aclada* grew well at 4 °C via the *in vitro* and *in vivo* tests.

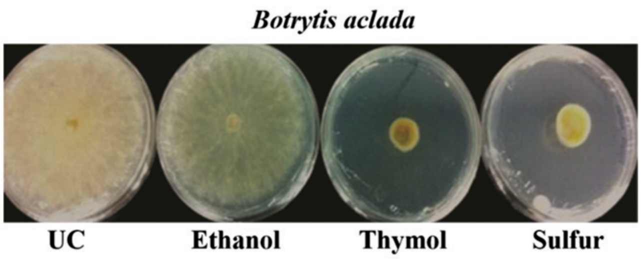

Figure 5. Investigation of antifungal activity of thymol. The concentrations of thymol and sulfur were 30 mg L^{-1} and 20 mg L^{-1}, respectively, for the treatments. Thymol treatment compared to the sulfur treatment resulted in superior antifungal activity against *B. aclada*. The antifungal activity of 30 mg L^{-1} thymol treatment showed significant effect for 7 days after *B. aclada* inoculation. UC, untreated control.

Table 2. Effect of thymol treatment on disease incidence in onion.

Treatment	Disease incidence rate (%) ± S.E[a] Incubation at 4 °C for 9 weeks
Untreated control	97 ± 0.7a[b]
Ethanol	50 ± 4.7b
30 mg L^{-1} Thymol	27 ± 1.4c

[a]S.E: standard error.
[b]Means followed by different letters were significantly different (LSD, $p = .05$).

postharvest disease incidence was greatly effective. In addition, the amount of residual thymol was well below the detection limit confirmed by GC-MS after long-term storage for 7 to 10 months in the low-temperature storage houses. After storage for 6 months, 70 onions in the untreated control group were infected by the low-temperature fungal pathogen. This number was obtained by visual observation. On the other hand, only three onions that were treated with thymol were infected by the low-temperature fungal pathogen. These results confirm that thymol treatment can result in a 96% reduction in fungal damage on onions during low-temperature storage (Table 5, Figure 6). The untreated control onions were sold after six months of storage to reduce further loss. However, the onions treated with thymol were sold after 10 months of storage with only a 17% loss of the onions. In the untreated control, a 20% loss had already occurred even after 6 months from the low-temperature storage.

We analyzed the thymol residues from the skin of the onions treated with the thymol solution fumigation after 10 months in the low-temperature storage houses. The results show that thymol residues were not detected on the onion samples (Supplementary Figure S3). Therefore, this indicates that onions treated with thymol do not

Table 3. Confirmation of disease incidence ratio after thymol fumigation treatment of the stored onions for 4 months.

	Disease incidence rate A(%)[b]			Disease incidence rate B(%)[c]: severe infection		
Treatment[a]	UC	Sulfur	Thymol	UC	Sulfur	Thymol
Average	33.3a[d]	17.8b	0c	10a[d]	1.5b	0c

[a]The onions in the 10 bags of 20 kg were treated with 30 mg L^{-1} of thymol or 20 mg L^{-1} of sulfur in the 14-ton cold storage container.
[b]Onions were infected only around root attached area.
[c]Whole onions were infected severely.
[d]The means followed by same letter are not significantly different between treatment (LSD, $p \leq .05$).

Table 4. Fungal colony counting existed in onion roots by each treatment.

	CFU[a]/g of root		
Treatment	UC[b]	30 mg L^{-1} Sulfur	30 mg L^{-1} Thymol
	4.9×10^5a[c]	1.5×10^5a	0.4×10^2b

[a]CFU denotes for colony forming unit.
[b]Untreated Control.
[c]The means followed by same letter are not significantly different between treatment (LSD, $p \leq .05$).

Table 5. Effect of thymol fumigation on fungal disease incidence of onion at low-temperature storage house.

	Number of infected onions in cold room				
	Botrytis aclada	*Fusarium proliferatum*	*Penicillium* spp.	Total[a]	Total infected onions (assuming whole room)[b]
Untreated Control	24	11	35	70	1540
10 mg L^{-1} Thymol	1	2	0	3	66

[a]The only sectors that could be accessible to persons, which cover one-twenty second sectors of whole area of low-temperature storage room (Figure 1).
[b]Expected number of total infected onions based on the assumption that the infection rate of onions in the whole room was similar regardless of sectors in a low-temperature storage house. Thymol fumigation using 10 mg L^{-1} thymol at low-temperature storage reduced the disease incidence to about 96% than that of the untreated control at 6 months after treatment.

Figure 6. The development of fungal disease in the commercial low temperature storage room at six months after fumigation of thymol. Mycelia grown on onion (A, B, and C) and not infected onion in the untreated room (D).

retain thymol residues, and thus the onions treated with the 10 mg L^{-1} of thymol were safe for consumption.

4. Discussion

Onion is a seasonal crop that requires long-term storage (more than 11 months) in low-temperature storage houses after each harvest. When the onions are harvested, most of the onions are not properly dried in South Korea because the season for the crop harvest overlaps with the rainy season. When stored under these conditions, onions are easily contaminated by fungal pathogens. If the onions get contaminated with mold, the infested onions stored for a long-term under highly humid conditions infect other healthy onions leading to huge losses; thus, it is important to control the spread of fungal pathogens early. Therefore, the control of fungal pathogens during low-temperature storage is one of the most important factors to extend the shelf life of onions.

According to our results on fungal occurrence in onions from low-temperature storage houses, *B. aclada, F. proliferatum*, *Penicillium* sp., and *R. oryzae* are the primary fungi in onion bulbs. Among them, gray mold caused by *B. aclada* is the most predominant economically important postharvest disease of onions. However, *B. aclada* was not isolated from the onions from the Garak commercial market. This might be due to the growth characteristics of *B. aclada* since it does not grow above 25 °C. The fungal isolates from the Garak commercial market were reported to be similar to our results. In their report, *B. aclada*, among the 233 fungal isolates was never recovered from the decayed samples of onions from the market [15].

The major cold storage pathogens of onions identified in this study through 18S rRNA gene sequencing was similar to that of *B. aclada* strain onion BC-15. PCR with gene-specific primers used by Nielsen et al. [11] and Coolong et al. [12] also identified the sample as *B. aclada* strain onion BA8 (data not shown).

To reduce the loss of infected onions by fungal disease at low temperature storage, we focused on the prevention of the predominant fungi *B. aclada*. The control of postharvest diseases using fungicides is a common practice by applying pesticides to onions in the fields before harvest, which reduces disease development during storage. Additionally, such postharvest disease has been controlled for many years by various pesticides or sulfur treatment technologies in low-temperature storage houses. However, the incidences of phytotoxicities caused by pesticides have increased. Moreover, the use of fungicides causes environmental problems because they do not decompose naturally. Notably, sulfur dioxide gas generated from sulfur treatment is the cause of metal corrosion and is harmful to human health. Therefore, many farmers avoid the use of this method in Korea; thus, safe and cost-effective alternative treatments to control postharvest fungal diseases need to be identified and implemented.

Dubey and Kishore [16] reported the antimicrobial activity of essential oils from *Melalleuca leucadendron*, *Ocimum canum*, *and Citrus medica* against *Aspergillus* species. It has been suggested that fruits and vegetables could be sprayed with or dipped into these oils to control postharvest diseases during storage [17–19]. Furthermore, these plant oils can be used very effectively to control pathogens because they are volatile [6,20].

Thymol is a major compound of thyme essential oils, and it is safe to be used for food preservation and as an additive in fruit juices [21]. It has been reported that fumigation of cherries with 30 mg L^{-1} of thymol resulted in a reduction of the gray mold *B. cinerea* to 0.5% from 35% in the untreated control [22]. It was also reported that gray mold on apples in storage was reduced by 38% using thymol treatment compared to the untreated control [23].

This study focused specifically on the application methodology of thymol for effective control of postharvest fungal diseases. To determine the appropriate application of thymol fumigation, we first tested the fumigation method by heating thymol powder. From the pilot experiment using a low-temperature 14-ton storage container, thymol residues were detected on the onion skins as well as the sides and bottom of the cold container after 30 mg of thymol powder was fumigated. The thymol residues of the inner containers had the highest concentration after 4 h following the fumigation treatment. The maximum concentration inside the container was 0.0211 mg L^{-1}, but the residual amount of thymol decreased at 4 h after the start of the fumigation treatment. In comparison, the residual amount of thymol on the onion skin per sample was 0.132–1.190 μg. The amount of thymol residues converted per 1 g of onion was 0.066–5.9 μg L^{-1} (data not shown). Through these results, we confirmed that thymol residues were detected around the fumigation equipment, indicating that thymol might not have spread evenly. Nevertheless, the thymol treatment showed a significant antifungal effect compared to the untreated control. However, in long-term storage of onions at low temperature, circulation is the most important factor because thymol must be spread evenly. Therefore, we concluded that the thymol granule fumigation method was not suitable for commercial scale applications.

Considering the above factor, a fumigation method using thymol solution was used on stored onions at low temperature. Thymol fumigation at a final concentration of 10 mg L^{-1} (weight:volume of the whole storage room) using thymol solution in low-temperature storage reduced the disease incidence to 96% compared to that of the untreated control at 6 months after the treatment. Onions treated with the thymol solution fumigation method had only a loss of less than 17% from fungal pathogens while maintaining the storage of the onions for 10 months in a low-temperature storage house. Compared to the untreated controls, the storage period of the onions treated with thymol was extended for more than three months. These results confirmed that thymol solution fumigation is a highly successful candidate for the control of fungal diseases during low-temperature storage. Additionally, since thymol residues from onions stored for 10 months were not detected, thymol solution fumigation could be a safe treatment method.

Thymol has been shown to be thermally safe up to 100 °C through the heat stability test [24]. In addition, there was no sterilization due to the methanol used in the dissolution of thymol. Our results also did not show any antifungal effects of ethanol on the postharvest disease of onion (Figure 4).

This study proposes that the fumigation treatment method using the thymol solution is ideal for fungal disease control of long-term storage crops such as onions. Based on our results, the mechanism for the antifungal effect on onions by thymol was presumed to be due to the phenol compound of thymol inhibiting the synthesis of chitin or β-1,3 glucans, which constitute the fungal cell wall.

With the results of reduced onion sprouts observed in the thymol-treated onions, it appears that thymol probably has a role in the regulation of physiological activity. Generally, when the crop respiration rate is high, the storage period is shorter because the crop ages faster. However, based on the results of this study, we hypothesized that the thymol-treated onion would have a lower respiratory rate than the untreated control, thereby reducing ethylene production. As a result, thymol can delay the ripening process after the onion harvest, and eventually, this mechanism can extend the onion shelf life.

Furthermore, the antifungal effects of thymol against fungal diseases caused by the distribution of grapes has been reported to be four times higher than that of linanool at the same concentration of 30 mg L^{-1} [13]. The authors reported that thymol could completely inhibit mycelial growth in *B. cinerea*. In this study, thymol solution fumigation (10 mg L^{-1}) significantly reduced the amount of onions infected with *B. acalda, F. oxysporum, Penicillium* spp., and other fungi in low-temperature storage. We also confirmed that the spray treatment using thymol solution was effective in controlling low-temperature onion pathogens. However, we suggest that the fumigation treatment using the thymol solution is more effective in increasing the circulation effect of thymol on a commercial scale.

B. aclada in this study is the most frequent fungal pathogen among the low-temperature storage pathogens. This study proposes a thymol solution fumigation method as a natural preservative and fungicide for effective disease management for various fruits and vegetables for long-term low-temperature storage in the near future. Furthermore, the thymol solution fumigation method could be commercialized for long-term storage to control postharvest diseases in various other crops.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by a special research grant [111071- 03-2-HD110] from Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fisheries (IPET). This work was partially supported by the Plasma Advanced Technology for Agriculture and Food (Plasma Farming) of National Fusion Research Institute of Korea (NFRI).

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